Long-term normothermic machine preservation of human livers: what is needed to succeed?

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Abstract

Although short-term machine perfusion (≤24 h) allows for resuscitation and viability assessment of high-risk donor livers, the donor organ shortage might be further remedied by long-term perfusion machines. Extended preservation of injured donor livers may allow reconditioning, repairing, and regeneration. This review summarizes the necessary requirements and challenges for long-term liver machine preservation, which requires integrating multiple core physiological functions to mimic the physiological environment inside the body. A pump simulates the heart in the perfusion system, including automatically controlled adjustment of flow and pressure settings. Oxygenation and ventilation are required to account for the absence of the lungs combined with continuous blood gas analysis. To avoid pressure necrosis and achieve heterogenic tissue perfusion during preservation, diaphragm movement should be simulated. An artificial kidney is required to remove waste products and control the perfusion solution’s composition. The perfusate requires an oxygen carrier, but will also be challenged by coagulation and activation of the immune system. The role of the pancreas can be mimicked through closed-loop control of glucose concentrations by automatic injection of insulin or glucagon. Nutrients and bile salts, generally transported from the intestine to the liver, have to be supplemented when preserving livers long term. Especially for long-term perfusion, the container should allow maintenance of sterility. In summary, the main challenge to develop a long-term perfusion machine is to maintain the liver’s homeostasis in a sterile, carefully controlled environment. Long-term machine preservation of human livers may allow organ regeneration and repair, thereby ultimately solving the shortage of donor livers.

isolated liver perfusion; liver physiology; liver preservation; liver transplantation; machine perfusion

INTRODUCTION

Liver failure is a health problem that affects many people worldwide, and for which researchers have been trying to find a treatment or cure for decades. In the 1950s, liver replacement therapy was first initiated by connecting porcine livers to the patient (1). Unfortunately, results stayed unsatisfactory, and when the safety and popularity of liver transplantation improved, experiments for liver replacement therapy came to a halt. Around the same time, the development of organ preservation machines began to evolve. Belzer et al. (2) developed a hypothermic pulsatile perfusion machine to preserve canine kidneys for 72 h. Also Brettschneider and Starzl (3) managed to perfuse canine livers successfully for 8–24 h before transplantation. However, as the machines were not superior to cold storage in graft survival and function, cold storage stayed the primary and preferred way of preserving organs (4).

Since the implementation of liver transplantation as a cure for end-stage liver disease, the disparity between supply and demand of donor livers has risen, necessitating the need to increase the use of extended criteria (high risk) organs (5). To mitigate the risks associated with extended criteria liver grafts, ex situ oxygenated machine perfusion (MP) has regained substantial interest in recent years (6).

Hypothermic oxygenated machine perfusion has been demonstrated to reduce ischemia-reperfusion injury of donor livers, leading to a reduction in postperfusion syndrome, early allograft dysfunction, and nonanastomotic biliary strictures posttransplantation (7). Normothermic machine perfusion (NMP) not only has the advantage that it enables to recover the liver at the donor hospital thereby shortening the exposure to cold ischemia, but also allows to test the liver’s viability before transplantation. This leads to an increase in the use of extended criteria livers without the risk of extra complications, which might result in lower waiting list mortality (8).

Although short-term NMP allows for resuscitation and viability assessment of high-risk donor livers, the organ shortage...
might be further remedied by perfusion machines suitable for long-term preservation. Ex situ MP for long-term (>24 h) perfusion of severely injured donor livers unsuitable for transplantation could potentially rescue such poor-quality livers, even those discarded following short-term NMP viability assessment. This recovery can be made passively by endogenous regeneration of the injured donor liver, leading to hepatocellular and cholangiocellular improvement. Alternatively, an initially unsuitable donor liver could become transplantable through exogenous stimulation of repair and regeneration pathways. For example, by the treatment with stem cells, immune modulators, antibiotics, small molecule interference against injury pathways, repair and regeneration pathways. For example, by the treatment with stem cells, immune modulators, antibiotics, small molecule interference against injury pathways, repair and regeneration pathways. For example, by the treatment with stem cells, immune modulators, antibiotics, small molecule interference against injury pathways, repair and regeneration pathways.

Several groups have already made successful progress in the development of long-term NMP (Table 1). This review summarizes the requirements and challenges for long-term, ex situ liver NMP which requires a system to mimic the human body by integrating multiple core physiological functions (Figs. 1 and 2).

MIMICKING THE HUMAN BODY

Heart and Vasculature

Pump.

A key prerequisite of mammalian physiology is the circulation of blood, realized by the heart’s pumping mechanism. Therefore, it is essential that the ex situ liver needs comparable circulation to maintain the internal environment including the delivery of O₂ and essential nutrients, and the removal of CO₂ and toxic waste products. The heart provides pulsatile flow with high pressure through the arteries to the organs, where the resistance of the extended capillary bed causes a decrease in pressure and a continuous flow returns through the veins back to the heart.

The advantages and disadvantages of pulsatile and continuous flow in organ perfusion systems have been studied in depth for decades. In 1978, Mavroudis (16) reviewed this topic for cardiopulmonary bypass machines, showing that pulsatile flow improved kidney function, lymph flow, and capillary circulation, with a decrease in mean arterial pressure, total periphery resistance, and anaerobic cell metabolism. After 1978, studies continued with a variety of pumps and materials. However, there is still no consensus on which flow profile is optimal. Some studies show preserved and improved (micro)circulation and more homogenous perfusion with pulsatile flow, whereas other studies on cardiopulmonary bypass or isolated organ perfusion do not find any differences between pulsatile and continuous flow (17, 18).

As pulsatile arterial flow is more physiological and several studies show superior results in both perfusion of the capillary bed and higher O₂ consumption of the surrounding tissue, a pulsatile flow is often used for the arterial side and a continuous flow for the portal side.

Pumps are a necessary component of MP to generate a forward movement of the fluid. Most organ perfusion machines use roller or centrifugal pumps, which can generate continuous or pulsatile flow (14, 15, 19). Furthermore, there is still much debate about what kind of pump is best to maintain the perfusion solution, including blood (components), in optimal condition. Blood components, such as red blood cells (RBCs), can get damaged (hemolysis) when used in MP due to the mechanical forces. The extent of hemolysis is related to exposure to shear forces and the duration of exposure (20). Some studies have shown less hemolysis with centrifugal pumps compared with roller pumps, whereas others have shown no difference or the opposite (20).

In human physiology, the pressure in the hepatic artery (HA) is similar as in the aorta (±120 mmHg), whereas the pressure in the portal vein (PV) is 6–10 mmHg, and in the vena cava (VC) 2–4 mmHg (21). In NMP, these pressures differ between studies, with HA pressure range between 40 and 100 mmHg and PV pressure between 5 and 18 mmHg (22). These pressures are correlated with flows ranging between 100 and 450 and 660 and 1,500 mL/min in the HA and PV, respectively (22). This is comparable with the total hepatic blood flow rate in human adults, which ranges from between 1,500 and 1,900 mL/min depending on liver weight, with an arterial-to-portal venous flow ratio of 0.58 (21). The hepatic arterial buffer response also persists in ex situ liver MP, indicating that the regulatory ability of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow is maintained (23). Because of the many differences between the perfusion experiments, it is difficult to say which pressures are optimal. However, in clinical MP it remains a balance between the lowest possible pressures to avoid unnecessary shear stress combined with sufficient flow rates to achieve adequate and homogenous perfusion.

Thus, for long-term NMP, shear stress in the perfusion circuit should be minimized, while maintaining adequate pressures and flow characteristics to achieve homogenous perfusion.

Tubing.

The heart is useless without the vessels leading the blood to the right places. In the body different kind of blood vessels exists: arteries, capillaries, and veins. They all have their own characteristics and function (24). Arteries have a ticker, elastic wall, are in general smaller in diameter than veins and are used to move the blood from the heart to the other organs. Capillaries consist of a single cell layer to facilitate the exchange of O₂, nutrients, and waste products inside an organ or tissue. Veins have a thinner wall and valves to facilitate the flow of blood back to the heart and function as a reservoir of the main amount of blood of the body. The currently used perfusion machines often do not distinguish in tubing between the HA and PV. Only the diameter of the tube is sometimes different, with a wider tube being used for the PV (12, 25). Capillaries exist inside the liver and do not have to be simulated.

It might be important to consider the length and the diameter of the tubing. The perfusion pressure and flow in the tubing can be described by the Hagen–Poiseuille equation

\[ \Delta P = \frac{8 \mu L Q}{\pi r^4} \]

where \( \Delta P \) is the pressure difference (in Pa), \( L \) is the length of the tube (in m), \( \mu \) is the viscosity of the perfusate (in Pa.s), \( Q \) is the flow rate (in m³/s), and \( r \) is the tube radius (in m). This equation shows the importance of the length and, especially, the diameter of the tubing on flow and pressure in the
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<tr>
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<th>Bral et al. (14)</th>
<th>Eshmuminov et al. (15)</th>
</tr>
</thead>
<tbody>
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<td>Porcine</td>
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<td>Cannulation</td>
<td>HA: 10-gauge</td>
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<td>HA: 10-gauge</td>
<td>HA: 10-gauge</td>
<td>HA: 7-Fr catheter</td>
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<td>(Hb 20–30 g/L)</td>
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<td>Methylprednisolone</td>
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<td>0.5 g</td>
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<td>Priming: 4,000 IU</td>
<td>Priming: 10,000</td>
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<td>IU of heparin</td>
<td>20,000 IU of</td>
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<td>During perfusion:</td>
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<td>During perfusion:</td>
<td>heparin</td>
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<td>Heparin bolus of 3.0 kIU every 4 h or heparin infused at 500 IU/h and adjustable to maintain an ACT &gt; 300 s</td>
<td>500 IU/h</td>
<td>830 IU/h of heparin</td>
<td>1,000 IU/h of heparin</td>
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</thead>
<tbody>
<tr>
<td>Other supplements</td>
<td>Priming: 9.2 mmol of CaCl₂, 20 mmol of NaHCO₃</td>
<td>Priming: 10% CaG 20 mL, 8.4% NaHCO₃ 20 mL</td>
<td>Priming: 10 mL CaG 10%, NaHCO₃ 20–50 mmol</td>
<td>Priming: FFP 2 units, 500–550 mL, CaG, NaHCO₃</td>
<td>Priming: FFP (± 0.8 L), albumin 20% solvent, NaHCO₃, CaG 20 mL</td>
<td>Priming: FFP (± 0.8 L), albumin 20% solvent, NaHCO₃, CaG 20 mL</td>
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<tr>
<td>Nutrients and hormones</td>
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<td>Glycemic control</td>
<td>Additional glucose or insulin infused to maintain glucose within a range of 4–10 mmol/L</td>
<td>Insulin 2.5 IU/h</td>
<td>After 4 h, glucose levels were kept within the desired range by algorithm-controlled automatic adjustments of on/off times of nutrition administration and insulin perfusion flow rates based on manual blood glucose measurement</td>
<td>Insulin 4 IU/h</td>
<td>Insulin 2 IU/h</td>
<td>To maintain a physiological blood glucose level (3.5–6.5 mmol/L), insulin ranged between 0.02 and 0.45 IU/mL and glucagon ranged between 0.02 and 0.5 IU/h.</td>
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<tr>
<td>Skin</td>
<td>Liver placed in an intestinal bag suspended in saline in a sterile perfusion chamber with a separate 800 mL reservoir.</td>
<td>Organ receptacle into a reservoir in which the organ is immersed.</td>
<td>Organ chamber with a separate 1,500 mL blood reservoir.</td>
<td>Container with separate reservoir.</td>
<td>Liver was placed on a silicone mat, which was fixed on the edges inside the organ chamber. An inflatable balloon beneath the mat connected to an air oscillator to induce oscillating movement of the liver 15 times per minute.</td>
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<td>Organ chamber</td>
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<tr>
<td>Antibiotics</td>
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<td>Cefotaxime 1g</td>
<td>Cefuroxime 750 mg</td>
<td>Cefotaxime 1g</td>
<td>Cefotaxime 750 mg</td>
<td>Priming: Piperacillin-Tazobactam 2.2 g</td>
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<td>During perfusion: Cefotaxime 1g/24 h</td>
<td>Vancomycin 0.5 g</td>
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<td>Vancomycin 0.5 g</td>
<td>During perfusion: Cefotaxime 750 mg/24 h</td>
<td>During perfusion: Piperacillin-Tazobactam 2.2 g/24 h</td>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Target flow</strong></td>
<td>HA: 300 L/min PV: 1.5 L/min VC: 1.8 L/min</td>
<td>Total: 1 mL/min/g tissue HA: 0.25 mL/min/g tissue PV: 0.75 mL/min/g tissue</td>
<td>No data</td>
<td>No data</td>
<td>HA: mean maximum of 0.6 L/min</td>
<td>HA: mean maximum of 0.6 L/min</td>
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<tr>
<td><strong>Target pressure</strong></td>
<td>HA: 85–95 mmHg PV: 5–10 mmHg VC: 0–3 mmHg</td>
<td>HA mean: 70–105 mmHg; PV: 3–5 mmHg (5–15 mmHg accepted during reperfusion to replicate the higher PV pressure encountered in patients with cirrhosis)</td>
<td>No data</td>
<td>HA: mean 80–100 mmHg PV: mean 8–12 mmHg</td>
<td>HA: MAP &gt; 65 mmHg PV: 5–10 mmHg VC: 0–2 mmHg</td>
<td></td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>38°C–39°C Heat exchanger</td>
<td>38°C Heat exchanger</td>
<td>37°C Heat exchange via inline Peltier heating elements.</td>
<td>36°C–37°C</td>
<td>39°C</td>
<td>Human: 34°C</td>
</tr>
<tr>
<td><strong>Brain and nervous system</strong></td>
<td>Pressure-monitoring cannula were placed in the VC (directly) and PV. Arterial pressures were measured directly from the arterial limb of the circuit. Continuous pressure monitoring was achieved using a transducer and a digital monitor. Pressure transducers, and flow probes. A gate clamp was used to obtain a target arterial pressure. Ascites collected in the sump of the organ chamber bowl was automatically returned to the circuit.</td>
<td>Continuous pressure monitoring was performed</td>
<td>Continuous pressure monitoring was performed</td>
<td>The centrifugal pumps were computer controlled to maintain the desired HA and PV pressures.</td>
<td>Flow sensors and pressure sensors measured flow and pressure. The control system aimed to maintain a desired flow in the PV and the desired pressure conditions in the HA. Also, an automated system for injecting a vasoconstrictor or vasodilator is used. Three individual gas-flow controllers for O₂, N₂, and CO₂ were controlled. An online blood-gas sensor continuously measured P_O₂, P_CO₂ and pH.</td>
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</table>

Only NMP > 24 h from several groups are listed, not from separate articles to maintain a clear overview. The articles chosen are the ones that describe the most of their setup. Some elements differ between the studies because of different end points investigated. However, the main elements are often similar. ACT, activated clotting time; CaCl₂, calcium chloride; CaG, calcium gluconate; FFP, fresh frozen plasma; Fr, French; HA, hepatic artery; Hb, hemoglobin; HT, hematocrit; ID, internal diameter; NaHCO₃, sodium bicarbonate; PCO₂, partial carbon dioxide pressure; PO₂, partial oxygen pressure; PV, portal vein; PVC, polyvinyl chloride; RBCs, red blood cells; TPN, total parenteral nutrition; UDCA, ursodeoxycholic acid; VC, vena cava.
Another important equation is the Darcy–Weisbach equation for maintaining a laminar flow

$$Re = \frac{\rho v d}{\mu}$$

where $Re$ is the Reynolds number, $\rho$ is the fluid density (in kg/m³), $v$ is the mean flow velocity (m²/s), $d$ is the diameter (in m), and $\mu$ is the viscosity of the fluid (in Pa.s). When the $Re$ gets too high (>2,400), flow will become turbulent, which changes the flow-pressure relationship (24). This can be physiological, but can also cause damage to the perfusate, such as hemolysis (20). The most optimal length and diameter of the tubing for the HA and the PV, in combination with the different perfusion fluids, have not been established yet.

Several kinds of tubing exist, all with their unique properties, but not all are suitable for long-term organ MP preservation. Most medically used tubing is made out of polyvinyl chloride mixed with plasticizers for obtaining flexibility (26). The plasticizer di(2-ethylhexyl) phthalate was commonly used, but di(2-ethylhexyl) phthalate tends to migrate from the plastic when exposed to lipid and protein-containing substances, such as blood and albumin (26, 27). Patients or isolated organs exposed to these tubes can take up this material, which is a concern due to its potential toxic and inflammatory effects (28, 29). Spallation (i.e., the detachment of polymer fragments) cannot only cause toxic effects but can also cause microemboli (30). This can be of added concern in long-term organ perfusion due to the duration of exposure. Other plasticizers, such as tris(2-tthylhexyl) trimellitate, have replaced di(2-ethylhexyl) phthalate and appear to cause less spallation (26, 31).

Not only the material of the tube is essential, but also the coating of the inside of the tube. A tube can be coated with a thin layer of substrate to prevent damage to the tube or to the perfusate. The coating can minimize spallation, and also reduce activation and loss of granulocytes and platelets, with synthetic polymer coating performing better than heparin coating in reducing inflammatory responses (20, 32–34). Studies have shown decreased complement activation and inflammation in coated versus noncoated tubing, whereas others did not find a difference (32, 33, 35). However, heparin-coated tubing is generally regarded as more biocompatible and widely used in extracorporeal circuits (34).

Finally, one can opt for a cannulated VC or not. The VC has normally a low pressure, between 2 and 4 mmHg, to create a pressure gradient and improves the homogeneous flow through automated feedback loops.
of the liver (21). The advantage of a cannulated VC is the enhanced possibility to accurately monitor all pressures and flows going through the liver (36). However, the downside is the possible obstruction and increasing pressure inside the liver when a pump mediates the outflow (11, 36). An advantage of a not cannulated VC is that the outflow pressure in the VC is always low, but there is less monitoring available (11). With a cannulated VC, there is always some leakage from the liver itself or the vessels, which needs to be drained and returned to the reservoir. This often requires an extra (roller) pump (12). Also, it remains to be established if the physiological, triphasic flow pattern of the VC is required to optimize liver perfusion during ex situ MP ("see Diaphragm movement").

In summary, tubing for long-term liver MP preservation has to fulfill several criteria. It should be made out of a material such as medical graded flexible polymer, which is strong and should be coated with a synthetic polymer or heparin coating to minimize spallation and increase biocompatibility. The tubing should have the right length and diameter to maintain a laminar flow, stable pressure, and minimize damage to the perfusate.

**Lungs**

**Oxygenators.**

During ex situ liver NMP, the metabolic demands are normalized to physiological levels, resulting in the need for artificial lungs to provide oxygenation and CO₂ removal to support these demands (37).

Different types of oxygenators exist, but currently the hollow fiber microporous polypropylene membrane oxygenators are most often used in clinical practice (38). These devices have exceptional surface-area-to-volume ratios, which allow them to quickly oxygenate and ventilate large amounts of blood/perfusate. However, because the material is microporous, it can absorb lipoproteins and become hydrophilic. This can result in plasma leakage inside the gas compartment, which subsequently can affect the performance of the gas exchange (39). These hollow fiber microporous polypropylene membrane oxygenators can be used for short-term (6–8 h) perfusions; however, for long-term use they may not be reliable. For long-term use, hollow fiber plasma-tight polyethylene diffusion membrane oxygenators are often used (38). These membranes have lower gas transfer capacity than the microporous polypropylene membrane in the short-term oxygenators, but become hydrophobic which diminishes plasma leakage. This makes these oxygenators suitable for long-term (5–30 days) use (38, 39).

Oxygenators are available in different sizes, aimed at providing oxygenation from newborns to adults. The best size to use for extended NMP for livers depends on the liver weight and the flows used (see Pump). In general, when a separate oxygenator is used for the HA and PV, a newborn size oxygenator will usually be sufficient. However, when only one oxygenator is used, a pediatric size oxygenator (or larger) is required (38).

The liver has a different vasculature than most organs due to its dual blood supply. The artery delivers maximally

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**Figure 2.** Characteristics of the different elements required for long-term liver normothermic machine preservation. The figure summarizes the required components of a perfusion device to allow long-term normothermic machine preservation.
oxygenated blood. However, the PV contributes substantially to oxygenation of the liver (+50%) due to its high flow and high O$_2$ saturation (80%–85%) (21). This dual blood supply would suggest that the PV system also has to be oxygenated, which is the case in some perfusion machines (13, 40, 41). On the other hand, it is also important to avoid hyperoxenation in the PV, as this leads to increased resistance and reduced flows in both the PV and the HA (42–44). It has been suggested that the endothelium plays a central role in the underlying mechanisms which causes this vasoconstriction due to hyperoxia, although the true mechanism is still unknown (43, 44). To avoid hyperoxia in liver perfusion, an arteriportal shunt is sometimes used to mix blood from the oxygenator and blood from the VC outflow to supply more physiologically oxygenated blood to the PV (42, 43). Physiological values of a partial pressure O$_2$ in a range between 9 and 13 kPa would be advised for the HA with an O$_2$ saturation of 80% for the PV (43, 44).

Besides delivery of O$_2$, the removal of CO$_2$ is also essential. An increase in CO$_2$ can cause a decrease in pH. During normothermia, the goal is to keep the pH as physiological as possible (pH 7.35–7.45). For balanced acid-base homeostasis, carbonic anhydrase plays a crucial role in the body as it facilitates efficient CO$_2$ transport by catalyzing the reversible hydration of CO$_2$ to bicarbonate and a proton. Accordingly, it also facilitates the supply of HCO$_3^-$, which is important for cholangiocyte function and bile production (45, 46). Bejaoui et al. (47) have described down regulation of carbonic anhydrase II after cold ischemia in steatotic rat livers, and the addition of bovine carbonic anhydrase II protected these livers against cold ischemia-induced injury and was associated with improved liver function after reperfusion.

In summary, normalized metabolic rate of the liver during NMP makes the use of oxygenators essential. For long-term perfusion, hollow-fiber plasma-tight polymethylpentene diffusion membrane oxygenators have to be used to stay functional during the whole extended perfusion. Because of the dual blood supply of the liver, with a lower physiological O$_2$ saturation in the PV than in the artery, these vessels require different blood/perfusate supplies with different O$_2$ saturations.

**Diaphragm movement.**

Inside the body, the liver is continuously exposed to movement from surrounding tissue as it is positioned above other intra-abdominal organs and suspended under the diaphragm. With each breath, the diaphragm moves up and down, intermittently causing positive and negative pressure on the liver while doing so, leading to expansion and compression of the liver’s vasculature. Lack of this diaphragm movement can result in pressure-induced necrosis and inhomogeneous perfusion of the liver during MP (13, 15, 42). For short-term NMP, this appears not to be a problem, but with an extended duration this could result in unreversible liver damage.

In 1968, Abouna (48) was the first to apply intermittent positive pressure in an extracorporeal liver perfusion system to mimic respiratory movements. These rhythmic movements produced a more homogeneous perfusion in the liver due to oscillations in hepatic venous pressure. Furthermore, the color, consistency, and function of the liver remained stable during perfusion up to 10 h, without internal edema formation (48).

Other groups explored the effect of liver movements during perfusion and found a 10%–25% increase of portal blood flow at a constant portal perfusion pressure, a decrease in PV pressure, and consequently a decrease in calculated vascular resistance. These movements resulted in improved homogeneous perfusion, and maintenance of liver architecture and function after perfusion for up to 24 h. In addition, bile production was higher, and bilirubin excretion was improved (42, 49).

Although subsequent experiments demonstrated the advantage of rhythmic movements, it has not been (widely) used in perfusion systems for human liver MP (50–52). This is probably due to technical reasons. Only recently, a perfusion machine with liver movement by mimicking diaphragm movement has been developed (15). Livers perfused within this machine showed homogeneous perfusion, metabolism, and no pressure necrosis.

These studies indicate that mimicking physiological diaphragmatic movement results in a more homogenous perfusion, reduced pressure necrosis, and improved oxygenation, all critical to achieve long-term liver MP.

**Kidneys.**

During NMP, normalized metabolic activity will lead to disturbance in the physiological environment over time. The kidneys are partly responsible for maintaining a stable internal milieu in the body. The rationale for using an artificial kidney in isolated liver MP is to maintain physiological pH by removal of nonvolatile acids, as well as maintain electrolyte, and osmolarity levels by removal of metabolic waste products during NMP. The implementation of a dialysis system for isolated liver perfusion has already been described as early as 1954 by Long et al. (53). Despite this, most modern perfusion machines do not have a dialysis system to mimic kidney function.

Perfusion of rat livers up to 8 h with an incorporated dialysis system showed lower potassium levels, a more physiological pH, and lower glucose and urea levels. Bile production was also higher, alongside less centrilobular necrosis and sinusoidal dilatation (54, 55). Dialysis with a closed-loop dialysate circuit (dialysate reservoir which is also used as supplement fluid) can be used for relatively short perfusions (<24 h), with or without changing the dialysate (54, 55). When the dialysate is not changed, the mechanism is based on plasma volume expansion, which will only be effective for a short perfusion time. Changing the dialysate will make more prolonged perfusion possible. However, this can result in fluctuating perfusate content, such as levels of urea (55).

Perfusion of isolated porcine and human livers has also shown disturbances of the physiological content of the perfusate by the accumulation of glucose as well as metabolic products, such as urea and lactate, which resulted in a rise in osmolarity and acidosis (56–58). Since the liver can normally produce hundreds of millimoles of urea per day, it is evident that to prevent extreme urea levels and therefore extreme osmolarities, urea must be removed during multiday perfusion. With a dialysis system added to the circuit, the perfusate pH, electrolytes, and osmolarity can be maintained near physiologic values, even up to 7-day of perfusion (15, 51, 58).
Other advantages of a dialysis system are the control of hematocrit by adjustment of the amount of substitution fluid, and possible manipulations of the content of the perfusate, such as adjustments of calcium to a lower physiological value, to inhibit elements that trigger ischemia-reperfusion injury (15, 57, 58). Several different dialysis systems have been used for the perfusion of porcine livers; however, for isolated human livers, continuous dialysis systems have mainly been used (15, 58).

Another technique to stabilize the environment is the use of a natural kidney. Several studies showed that adding a porcine kidney to an isolated porcine liver in a perfusion system makes the environment more physiological. Glucose values normalized, pH, electrolytes, and urea remained more stable. Furthermore, a better-preserved architecture of the liver with lower oxidative stress and reduced inflammatory cytokines were observed (59, 60). A combined ex situ perfusion of a human kidney and liver from (ideally) the same donor, however, has not yet been reported. Another theoretical possibility would be combining an animal kidney, such as a porcine kidney, with a human perfused liver. Nevertheless, this may give rise to other problems, such as immunological complications, the risk of zoonosis, and may require genetic engineering (61). Therefore, using an artificial kidney for long-term NMP seems the most feasible.

A dialysis system is not always implemented and needed in a short-term MP. Nevertheless, a more stable environment is created by integrating a dialysis filter to keep the pH, electrolyte, and osmolarity levels more physiological by removing waste products, toxins, and other metabolites. Also, the architecture of the liver is better preserved with decreased oxidative stress and inflammatory cytokine production. When long-term liver MP preservation is desired, a continuous dialysis system will be mandatory for the liver to remain vital.

Blood and Bone Marrow

**Oxygen transport.**

Red blood cells are crucial for the body as they carry O₂ to the tissues and remove CO₂. At present, the majority of perfusates used in NMP are RBC-based, taking advantage of the naturally occurring hemoglobin and the erythrocyte’s metabolism as an efficient O₂ and CO₂ transport system. However, due to the high costs, scarcity, and risk of transmitting blood-borne infections of human blood products, alternative hemoglobin-based oxygen carriers (HBOCs) such as HBOC-201 (Hemopure, HbO2 Therapeutics LLC), which is bovine-derived polymerized free hemoglobin, have also been used (40, 62).

Bodewes et al. (37) have described which alternative O₂ carriers can be used for MP. These alternative carriers include HBOC-201, hemoglobin vesicles, Hemarina M101 (HEMO2life, France), and perfluorocarbons. All have their advantages and disadvantages, as described in Table 2, and are reviewed in detail elsewhere. General advantages are no need for blood type-matching, better availability, better storage conditions, and less risk for blood-borne infections. Some disadvantages are the lack of the natural methemoglobin-reductase in HBOC-201, that causes formation of methemoglobin, or the reduced O₂ content in perfluorocarbons because of the emulsion composition.

The viscosity of the perfusion fluid with an O₂ carrier can also affect the relationship between pressure and flow in the circuit and the liver, as is reflected by Eqs. 1 and 2. A viscosity that is too low can cause a turbulent flow, and a viscosity that is too high needs a higher pressure for the same flow.

For long-term NMP, an artificial O₂ carrier could minimize the risk for blood-borne infections, avoid blood type-matching, and has the advantage of a longer self-life. Also, RBCs are scarce and with the increasing development and use of NMP for several organs, this might become a problem. However, artificial O₂ carriers are currently not approved for clinical use, and their utility and safety remain to be established for long-term perfusion.

**Immune system.**

The liver itself plays an essential role in the immune system and contains many innate and adaptive immune cells (63). However, extracorporeal membrane oxygenation (ECMO), a cardiopulmonary bypass machine, results in an immediate inflammatory reaction with a rise in proinflammatory cytokines and activation of the complement system (64). The same reaction is likely to occur in MP, together with immune activation which already starts during warming up the liver to normothermia. This activated immune response must be controlled to prevent too much activation and avoid injury to the liver. Immune suppressive medication can be a solution, but is not commonly administered in liver MP. Only a few groups are giving immune suppressive medication during short-term NMP, whereas for >24 h it becomes more common. Markedly different doses are being used. Most often 500 mg methylprednisolone per perfusion per 24 h or 10 mg/L hydrocortisone are administered (11, 14, 15, 65). However, whether this is effective has not yet been clarified, nor have the possible harmful consequences.

Another important part of the immune system is the complement system. The liver produces the majority of the complement proteins, and is itself quite insensitive to complement attack (63). This is probably the main reason why the complement system is not much debated in liver NMP. However, it is implicated in both ischemia-reperfusion injury and in liver regeneration, which makes the balance of the complement system important. This means that complement factors may potentially play an important role during long-term NMP (63).

For long-term liver MP preservation, immune-suppressive medication might be necessary to prevent an overly activated immune system. Future research will have to show to what extent this will affect the perfused liver. Furthermore, the complement system requires further investigation to determine potential positive or negative effects during NMP.

**Coagulation.**

During NMP, activation of the coagulation cascade may potentially lead to formation of (micro)thrombi, causing areas of ischemia in the liver and possible obstruction of the liver microvasculature. A competent coagulation system is present when fresh frozen plasma (FFP) is used in the perfusion fluid. However, in plasma-free perfusion fluids, synthesis of relevant amounts of coagulation factors during NMP of
human liver occurs already after several hours (66). Coagulation may be activated by exposure of the perfusate to artificial materials used in the machine and potentially by the release or decryption of tissue factor by the liver (66–68).

To prevent this activation, anticoagulation strategies are used in medical devices, such as ECMO, but also in organ perfusion machines it is common to use an anticoagulant. Most often, anticoagulation is achieved by the addition of high doses of unfractionated heparin (67, 69). Although most groups performing liver MP use unfractionated heparin, there is still no consensus on the correct dosage (69). One drawback of heparin is that it requires binding to antithrombin or heparin cofactor II to function, and it induces release of tissue factor pathway inhibitor (70). For this reason, bivalirudin has been used as an alternative in ECMO treatment (67). Bivalirudin has a direct anticoagulant effect without the requirement for other proteins such as antithrombin (71). However, if bivalirudin is a superior anticoagulant than heparin for NMP still requires investigation. We have previously demonstrated that coagulation is not activated during 6 h of NMP, despite the synthesis of physiologically relevant concentrations of coagulation factors, suggesting adequate heparin anticoagulation (66, 72). It will, however, be mandatory to monitor activation of coagulation during longer periods of perfusion, using markers of activation of coagulation such as thrombin-antithrombin complexes and D-dimer levels. Also, assessment of heparin levels by anti-Xa tests will be helpful during long-term perfusions.

Most studies only focus on the anticoagulation part of the coagulation cascade. Nevertheless, the liver plays an important role in both pro and anticoagulatory components. It is not known yet if the synthesis of coagulation factors by the isolated liver and accumulation of these factors in the perfusate has a limit (66). In one study, factor V, brinogen, and factor VII appear to have reached a plateau when measured for several days, which may indicate that an equilibrium between synthesis and clearance of coagulation factors by the liver has been established. However, antithrombin exceeded the maximum measuring limit after only 3 days of perfusion (73).

Table 2. Overview of the advantages and disadvantages of different oxygen carriers (38)

<table>
<thead>
<tr>
<th>Oxygen Carrier</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Pharmacokinetics</th>
<th>Possible Toxicity in Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin in red blood cells (RBCs)</td>
<td>Within its physiological microenvironment</td>
<td>Immune-mediated phenomena</td>
<td>$T_{1/2} = 60$ days</td>
<td>ABO incompatibility</td>
</tr>
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<td></td>
<td>Human blood product</td>
<td>Blood-borne infection transmission</td>
<td>MW = 64 kDa</td>
<td></td>
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<td></td>
<td>Low methemoglobin production</td>
<td>Fragility</td>
<td>$[Hb] = 12–15$ g/dL</td>
<td></td>
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<tr>
<td></td>
<td>Dynamic shift of $O_2$-hemoglobin dissociation curve</td>
<td>Insufficient $O_2$ delivery at low temperatures</td>
<td>$P_{50} = 27$ mmHg</td>
<td></td>
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<td></td>
<td></td>
<td>RBC hemolysis during hypothermic machine perfusion</td>
<td></td>
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<td></td>
<td></td>
<td>Cross-matching difficulties</td>
<td></td>
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<td></td>
<td></td>
<td>Precious resource</td>
<td></td>
<td></td>
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<tr>
<td>Hemoglobin-based oxygen carrier-201</td>
<td>Easy $O_2$ release to tissue</td>
<td>Formation of Methemoglobin</td>
<td>$T_{1/2} = 20$ h</td>
<td>Systemic Vasoconstriction</td>
</tr>
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<td></td>
<td>Sterile and pyrogen-free</td>
<td></td>
<td>MW = ~250 kDa</td>
<td></td>
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<tr>
<td></td>
<td>Large temperature range ($4^°$–$37^°$C)</td>
<td></td>
<td>$[Hb] = 13$ g/dL</td>
<td></td>
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<tr>
<td></td>
<td>Less viscous than RBC</td>
<td></td>
<td>$P_{50} = 38–40$ mmHg</td>
<td></td>
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<td></td>
<td>Long shelf life: 3 years</td>
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<td></td>
<td>Compatible with all blood types</td>
<td></td>
<td></td>
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<tr>
<td>Hemoglobin vesicles</td>
<td>Absence of RBC antigens</td>
<td>Only used in animal models</td>
<td>$T_{1/2} = 2–3$ days</td>
<td>Release of free Hb can cause renal toxicity</td>
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<td></td>
<td>Smaller than RBCs</td>
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<td></td>
<td>Do not generate colloidsosmatic pressure</td>
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<td></td>
<td>Do not rupture</td>
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<tr>
<td></td>
<td>Long shelf life: two years</td>
<td></td>
<td></td>
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<tr>
<td>Heminis M101</td>
<td>Preliminary evidence in static cold storage</td>
<td>Only used in preclinical HMP and clinically in static cold storage</td>
<td>MW = 3,600 kDa</td>
<td>None reported</td>
</tr>
<tr>
<td></td>
<td>Large temperature range ($4^°$–$37^°$C)</td>
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<td></td>
<td>Simple gradient release $O_2$</td>
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<td></td>
<td>High $O_2$ affinity</td>
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<tr>
<td></td>
<td>Nonimmunogenic</td>
<td></td>
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<tr>
<td>Perfluorocarbons</td>
<td>High $O_2$ solubility</td>
<td>Formulated as emulsion, which reduces $O_2$ content</td>
<td>$T_{1/2} = 8–24$ h</td>
<td>Loss of vision</td>
</tr>
<tr>
<td></td>
<td>Inexpensive</td>
<td>Needs high $PO_2$ to maximize $O_2$ content</td>
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<tr>
<td></td>
<td>Obeys Henry’s law</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$O_2$ uptake and release insensitive to environment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Delivers sufficient $O_2$ below $20^°$C</td>
<td>$O_2$ content decreases with higher temperature leading to a mismatch above $20^°$C</td>
<td>$T_{1/2} = \infty$</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Inexpensive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$[Hb]$, concentration of Hb or its equivalent for other carriers; MW, molecular weight; $P_{50}$ = $O_2$ tension where 50% of Hb is saturated with $O_2$; $T_{1/2}$, half-life.
of coagulation factors in the perfusate. If there is indeed no feedback system inside the isolated liver to limit the synthesis of coagulation factors, the perfusate may have to be periodically refreshed. The progressive accumulation of coagulation factors may lead to significant complications, such as unacceptable increases in viscosity or even uncontrolled coagulation. Better understanding of the mechanisms regulating the synthesis of pro and anticoagulation factors may indicate ways to selectively inhibit this process. Alternatively, plasmapheresis or other modes to selectively remove coagulation factors could be explored.

In summary, a form of anticoagulation is essential for long-term liver MP preservation. However, whether unfractionated heparin is the best agent remains a question. Alternatives, such as Bivalirudin remain to be studied in preclinical and clinical settings. Also, if synthesis of pro and anticoagulation factors proceeds uncontrolled, a way has to be found to remove the accumulation of these factors to prevent complications. Nevertheless, the aim remains to completely anticoagulate the perfusate, which is now achieved by high-dose heparin.

Other substances.
The blood contains many more substances than just described. The perfusate composition is extremely important for liver perfusion, especially for long term.

Prostacyclin (epoprostenol) is a drug which is often used to obtain vasodilatation in the microcirculation in liver MP (8, 10, 56). Beside the vasodilatation effect, it is also a platelet function inhibitor. It has been shown that administration of prostacyclin can increase bile production, decrease liver injury markers, and preserves hepatic architecture compared with adenosine or perfusion without a vasodilator (75). However, not all groups use it and do not show any problems without the use of prostacyclin (14, 15, 40). This might indicate that other (endogenous) factors may facilitate vasodilatation.

Other main differences in the perfusate compositions are the use of plasma or plasma-free solution, and the use of colloid solutions (12, 13, 15, 76). However, the different perfusate compositions used do not appear to show major differences in liver perfusion outcome. So, what the best composition for long-term perfusion is, has to be studied when more of these perfusions will be performed.

Nutrients and Hormones
Nutrients.
In 1993, Schon et al. (77) demonstrated that the use of nutrients reduced the release of transaminases and improved the ability of the livers to metabolize ammonia in porcine liver perfusion. Today, most groups use some suppletion of nutrients. When nutrients are provided, they often include several compounds such as total parenteral nutrition for humans, glucose, and amino acids as well as multivitamins and trace elements (12, 15, 25, 41, 58, 78–81). However, dosing and the route of administration differ. Most groups supply nutrients continuously (12, 15, 25, 41, 58, 78, 79), whereas others only provide a bolus at the start (10, 80, 81). Ravikumar et al. (19) regulate the infusion of amino acids by the measured glucose levels. The benefit of adding lipids is not often discussed. Butler et al. add 50 mL of 10% lipid emulsion (10), but most groups do not report about lipid emulsion or do not supply it (8, 25).

Even though nutrients are almost seen as a necessary standard, it is not clear how much should be given. Relling et al. (56) point out that administration of too many amino acids might result in higher urea production, which can cause marked increase in osmolarity which may negatively affect liver function. This can be solved using an artificial kidney, but prevention of excessive urea production might be preferable. For short-term perfusions, this seems not to be a problem, but it will become quite important when perfusions are prolonged across several days. Much more research will be needed on this subject to find out what an isolated liver truly needs.

Bile salts.
Bile is a crucial substrate produced and secreted by the liver. It helps the body to remove toxic, and poorly water-soluble waste products, it is involved in transporting fats and liposoluble vitamins, and aids in cholesterol homeostasis (82). Bile production is also used as an important viability criterion for the quality assessment of a liver during NMP (83).

For bile production, bile salts conjugated with glycine or taurine (bile acids) are essential (82). Generally, <10% of the bile acids are excreted in feces, with the majority being reabsorbed during the enterohepatic recirculation (82). However, in isolated liver perfusion, the enterohepatic recirculation is absent and has to be simulated by replacing bile salts.

Imber et al. showed in porcine livers that the addition of bile salts is necessary for prolonged perfusion. After 20 h of perfusion, bile production started to drop from a maximum of 14.8 mL/h at 6 h to 1.7 mL/h after 40 h when no supplementation had taken place. Also, a 30% reduction in bile acid production was seen after 20 h of perfusion. With the addition of 70 mg/h taurocholate, bile production stayed around 8 mL/h (25). Javitt already described the positive effects of sodium taurocholate on bile flow and bile acid excretion in 1968 (84). Imber et al. are not the only group using taurocholate for porcine and human livers. Many groups supplement bile salts continuously with or without an initial bolus for perfusions of 3 h up to 7 days (15, 19, 25, 41, 58, 80). Most use taurocholic acid, however ursodeoxycholic acid (UDCA), and collection and reinjection of the bile salts into the perfusate to stimulate enterohepatic recirculation have also been used (15, 57). With taurocholic acid being a hydrophobic bile acid, it can enhance detergency resulting in liver and biliary epithelium damage (82). This would suggest that a bile salt other than taurocholic acid should be used to protect the liver. UDCA, which is more hydrophilic, has shown protective properties (82).

To maintain adequate bile production and composition during long-term liver MP preservation, bile salts should be administered during perfusion. The optimal composition, amount, and way of administration have yet to be determined. In particular, hydrophilic bile salts such as UDCA appear to be of interest because of their putative role to protect the biliary epithelium.

Hormones.
The liver is a key metabolic organ, controlled by insulin and other metabolic hormones. The release of glucose to the
circulation is partly regulated by the hormones insulin and glucagon produced by the pancreas. The liver itself uses mainly fatty acid oxidation as an energy source, although glucose is a necessary substrate for bile production (85, 86). Despite this, in human or porcine liver NMP, glucose is not routinely supplied after the start of perfusion, even in cases of perfusion up to 48 h (14, 76, 80, 81, 87). This is probably due to already high glucose levels during the first hours of perfusion (56). A decrease in perfusate glucose level appears to be a good sign of liver function, as hepatocytes take up glucose with subsequent conversion to glycogen (58). Some groups administer boluses or a continuous glucose infusion to keep the perfusate glucose level in a specific range (19, 65, 79). Others administer total parenteral nutrition meant for humans instead of glucose alone when the perfusate glucose drops below a particular value (8, 12, 88). One group supplied glucagon when glucose dropped below a certain threshold (15). On the other hand, insulin is almost always supplied during perfusion even when the high perfusate glucose levels in the first hours of perfusion appear to be resistant to insulin (8, 10, 12, 19, 56, 65, 87).

Although physiological glucose levels are between 4.0 and 6.5 mmol/L, a glucose level of between 4.0 and 10.0 mmol/L is generally considered desirable in diabetics (89). For liver MP, an optimal range has not yet been determined. Different perfusate glucose targets are currently being used (>2.2, 3.5–6.5, 4–10, <10, >10, or 12–15 mmol/L) (8, 10, 12, 15, 65, 79, 88). As the liver is normally not predominantly dependent on glucose itself, it may not be necessary to have high glucose levels present in the perfusate. This was also observed during 7-day perfusion. A glucose level of >10 mmol/L resulted in high insulin administration rates, activation of glycogen synthesis and excessive glycogen deposition in liver tissue. This was observed to a lesser extent with glucose concentrations around 4 mmol/L (15).

For short-term perfusion, glucose monitoring and supplementation of substances to keep glucose in a specific range can be done manually. However, for long-term liver MP preservation, an automated system is desired. Artificial pancreases with closed-loop systems for glucose regulation have been tested for over 40 yr, with some systems already being CE-marked or FDA approved for clinical use in patients (90). Most of these artificial pancreas devices use only insulin to control the blood glucose level.

Thus, the role of the pancreas in long-term liver MP preservation can be mimicked through closed-loop control of glucose concentrations. This can be achieved by using a glucose sensor and automatic injection of insulin and/or glucose, with a glucose level between 4 and 10 mmol/L appearing to be most appropriate target.

Spleen

The spleen forms an essential part of the human immune system. It initiates immune reactions and filters the blood of damaged red blood cells. This system is difficult to mimic, but some spleen functions might be achieved by a leukocyte filter.

In animal liver perfusions, whole blood has been used as the perfusion fluid, and in this case, leukocytes are part of the perfusate. In human liver perfusions, leukocytes are removed from perfusate components by the use of leukocyte-depleted RBCs. However, some residing leukocytes can also be transferred from the donor liver to the perfusion circuit and may possibly activate an immune reaction and cause graft dysfunction (91). For this reason, some isolated liver MP devices have an integrated leukocyte filter to eliminate circulating leukocytes, with the additional advantage that clots and microthrombi are also removed from the perfusate (14, 87, 92). Some groups only use a leukocyte filter before administration of the perfusate into the circuit (15, 57). Most, however, do not use a leukocyte filter or do not report using leukocyte depleted RBCs, especially in porcine studies (41, 65, 76, 79, 81, 88). This contrasts with other organs (kidneys, lungs, heart) where the use of a leukocyte filter or leukocyte depleted RBCs in the perfusate is almost always mentioned (93–95).

Another option or addition could be the use of the Cytosorb (Cytosorbents Corporation) adsorber, a device that can reduce excessive levels of cytokines. At present, it is mainly used and tested in septic patients and in cardiac surgery (96). In both settings, inconsistent results have been obtained. A reduction in interleukin-6 is often observed but without consistent clinical improvement (96). In addition, some experiments have been performed with the Cytosorb adsorber combined with an organ perfusion machine. During normothermic kidney perfusion, a reduction in inflammatory response (mainly interleukin-6, interleukin-8, prostaglandin E2, and thromboxane) and improved renal blood flow during perfusion was found. However, it did not show any effect on renal function (measured by creatinine clearance) (97). In ex situ lung perfusion for 12 h, cytokine removal showed improved airway pressure and dynamic compliance, decreased pulmonary edema, and improved electrolyte balance. Also, glucose consumption and lactate production were reduced (98). One of the major disadvantages of the Cytosorb adsorber is lack of selectivity, which may not only reduce inflammatory cytokines, but also reduce beneficial mediators and medication added to the patient or system, including antibiotics (96, 97).

A leukocyte filter seems important to remove leukocytes and decrease ischemia-reperfusion injury during long-term liver MP preservation. For the Cytosorb adsorber, the use in liver MP is questionable and needs to be further studied.

Skin and Abdominal Cavity

Organ chamber.

During perfusion, the liver requires an organ chamber that houses specific conditions, similar to those in the abdomen. It has to be warm, humidified, and surrounded by movements from other organs, such as the diaphragm and other abdominal organs, as described earlier.

The majority of organ chambers comprise a container that can be opened and closed by a removable lid and a construction where the liver is positioned either on a flat or concave surface, or is suspended in a fluid to prevent pressure necrosis and other injuries (11, 19, 42, 48, 78, 86). In most cases, the liver is placed with its diaphragmatic side down on a specific net or in an intestinal bag to allow access to the vessels and maintain its original anatomical shape as much as possible (11, 42, 48, 78). This functions well enough for short-term perfusion.
perfusion, but whether this is also sufficient for long-term liver MP preservation is yet to be determined.

**Sterile environment.**
In the human body, the liver is surrounded by tissues, fluids and, ultimately, covered by the skin. The skin is the main barrier for the human body to prevent contamination with specific microorganisms including bacteria and fungi and keep most of the body sterile. With isolated liver perfusion, where the liver is taken out of its sterile environment, it is crucial to prevent contamination of microorganisms. Especially, because the NMP perfusate is an optimal culture medium for microorganisms, due to the normothermic temperature and the nutrition added to the perfusate.

In most normothermic isolated liver perfusions, antibiotics, such as cefotaxime, cefazolin with metronidazole, cefuroxime, piperacillin-tazobactam, vancomycin, gentamicin, or imipenem-cilastatin are standard and provided at the start of perfusion, being repeated every 24 h (10, 14, 15, 19, 41, 65, 80, 81, 87, 88, 96, 99). The question is if the use of very broad-spectrum regimens is necessary. Bruinisma et al. examined the bacterial contamination of *Staphylococcus epidermidis* and *Staphylococcus aureus* during (sub)normothermic machine perfusion (28°C) and found a sustained bacterial growth without the use of antibiotics. They also showed that 1,000 mg/L cefazolin prevented this bacterial growth without adverse effects on porcine kidney endothelial cells (100). Eseshimov et al. also explored the best preventative strategy during 7-day NMP and found microbial contamination, mostly from an external source, probably due to operating the perfusion machine. They also showed that continuous infusion of piperacillin-tazobactam was preferred over a daily bolus to maintain a stable concentration (101).

A case of recipient sepsis due to *Escherichia coli* transmission after clinical liver NMP has already been described. The *E. coli* was resistant to cephalosporin and cefuroxime, which were added to the perfusate before perfusion (102). This indicates that awareness of possible contamination of a microorganism after NMP is even more important than the addition of antibiotics. Beside systemic contamination, the bile can also become contaminated. Bactobilia is a known problem in patients after liver transplantation and can cause cholangitis and even sepsis. This can often be managed with piperacillin-tazobactam, ciprofloxacin, or amoxicillin-clavulanic acid. However, if these antibiotics should be given as prophylaxis remains debatable (103).

Sterility is also a challenge in patients treated with ECMO. Nosocomial infections are a common problem, mainly due to invasive devices, frequent entry into the circuit for laboratory assessments and drug administration, and because these patients are often immunosuppressed (104). ECMO duration is the only predictor for acquiring a nosocomial bloodstream infection and the risk increases with prolonged duration (104). This principle may also apply to isolated liver perfusion, and long-term perfusion will likely result in a higher risk of contamination.

The most common organisms seen during ECMO are *coagulase-negative staphylococci, Candida* spp., *P. aeruginosa, S. aureus, Enterococcus, Enterobacter* spp., and *S. maltophilia*, which are also common organisms for the majority of the non-ECMO device and procedure-related healthcare-associated infections and were also found during 7-day liver perfusions (101, 104, 105). Fungal infections are not often seen. However, they carry an increased risk of hospital mortality if they do occur (106). For this reason, strategies similar to those advised for ECMO to prevent catheter infection can also be applied to isolated organ perfusion. Suggestions from The Extracorporeal Life Support Organization to maintain the ECMO circuit sterile is to: 1) use needleless hubs for all connections and access sites in the circuit; 2) only administer continuous infusions to the circuit (to minimize “breaking” the sterility of the lines); and 3) avoid use of prophylactic antibiotics because there are no data to support the routine use of continued antibiotics for patients on ECMO and prophylactic antibiotics may increase the risk for resistant strains and potential yeast overgrowth (107).

Another concern for bacterial growth is the material that is used in MP. The synthetic surface of, for example, the tubing may become contaminated with bacteria (108).

Sterility is essential for long-term liver MP preservation. To maintain sterility, it might be best to follow The Extracorporeal Life Support Organization guidelines because of their experience with long-term perfusions with adaptations specifically for MP. Although antibiotics are standard supplied in liver perfusion, in long-term liver MP preservation they should be used with caution to decrease the risk for resistant strains inside the liver, which could be transferred to the immune-compromised recipient. More important is the awareness that contamination with a microorganism is possible after liver NMP.

**Brain and Nervous System**
A healthy human body is the ultimate well-oiled machine with all the organs working together and helping each other to perform their function. This is regulated partly by the organ itself (such as the heart and auto-regulation of the kidneys) but also organized from a higher level by the brain and the nervous system.

After heptectomy, the liver gets deprived from its neural innervation and the liver-brain axis is distorted. This appears not to have major detrimental effects on the liver graft, in term of bile secretion and hepatic blood flow. However, reduced glycemic control with insulin resistance and reduced stimulation of hepatic progenitor cells are adverse events following the removal of neural innervation (109). In addition, vagus nerve stimulation has shown to decrease ischemia-reperfusion injury in rats (110). Even though vagotomy has no major adverse effect after liver transplantation in humans, it might cause extra liver damage in a liver during long-term NMP. Therefore, it may be beneficial to stimulate neural innervation.

Most liver MP are not entirely automated, most likely because the majority of clinical liver NMPs are between 4 and 24 h (11, 40, 41, 56, 65, 99). To perfuse an isolated liver in a machine for several days, some procedures will have to be automated to maintain a good/physiological environment for the liver, such is done with the 7-day perfusion machine (15).

The main elements which have to be automated are the flows and pressure through the veins and artery, the
temperature, and the perfusate composition. These elements need online sensors with an incorporated feedback loop to maintain the desired values. Ideally, such a system should be monitored from a distance with an integrated interface, and control values might be adjusted remotely.

**Control of pressure, flow, and temperature.**

Even though blood flow rates and pressures differ between studies in human livers (“see Pump”), these parameters are vital for MP (22). Most machines use a flow- and pressure transducer/probe connected to a computer or monitor to continuously measure flow and pressure (11, 15, 58). Eshmuninov et al. (15) also used a feedback loop for automatic injection of vasoactive medication to maintain a certain pressure and flow. Perfusion machines can be either pressure-controlled or flow-controlled. The flow is dependent on vascular resistance (Eq. 1), so the advantage of pressure-control is that the pressure can never get too high, which will protect the liver from shear stress in the vessels, which in turn prevents endothelial cell damage.

The temperature for NMP is often set between 35.5°C and 37.5°C for human livers. Eshmuninov et al. (15), however, used a temperature of 34°C (which in essence, is subnormothermia) for their 7-day experiments. The temperature must stay stable during the long-term perfusion. This can be achieved by several methods (heat pump concept, thermoelectric heating by Peltier-effect, principle of Joule heating, thermal radiation), but is most practically done by regulating the perfusate temperature instead of temperature of the liver itself. Because oxygenators are essential for NMP, the use of oxygenators with an integral heat exchange circuit and a temperature adjustable water reservoir is the most straightforward method to maintain a specific temperature while preserving perfusate sterility. Also, the body temperature of a human has a circadian rhythm, which may positively affect the liver’s metabolism as well (111). This implicates that the temperature should be adjustable during the whole perfusion period to achieve a physiological environment. To maintain a stable temperature during long-term perfusion, temperature sensors are needed to control thermoregulation, although a sensor within the water circuit may suffice, given the excellent heat-exchange characteristics of oxygenators with a water circuit.

Altogether, a pressure-controlled machine would be preferred above a flow-controlled machine to maintain specific flows while avoiding the risk of excessively high pressure, preventing liver damage due to shear stress. Likewise, control of the perfusate temperature during long-term MP preservation is important.

**Monitoring of perfusate composition.**

The perfusate composition supplies the liver with its metabolic needs and allows removal of waste products. To maintain this finely balanced physiological environment, monitoring the content of the perfusate is fundamental.

It is clear that PO2 and PCO2 are essential determinants of liver metabolism. Continuous measurement of these values and pH is often measured by electrochemical and optical sensors. Several commercially available systems have been developed for continuously measuring these parameters, such as the CDI Blood Parameter Monitoring System (CDI-3M Healthcare, Tustin, CA), the Optex Biosentry System (Optex Biomedical, The Woodlands, TX), and the PB System (Puritan Bennett Corp., Carlsbad, CA) (112). Integration of a continuous monitoring system into the liver perfusion machine will be required to maintain a stable environment for the liver in long-term liver MP preservation.

For short-term clinical NMP, most often (point-of-care) blood gas analyzers are used to intermittently assess the composition of the perfusate, including lactate and electrolytes (14, 76, 99).

Maintaining a physiological environment does not stop with measuring specific values alone. For long-term liver MP preservation, automated feedback loops should be integrated into the perfusion system to correct these specific values and maintain the desired physiological environment.

### MONITORING LIVER FUNCTION AND VIABILITY ASSESSMENT

For a liver on long-term NMP, it is of vital importance to monitor its function during perfusion to adjust the machine’s settings and modify the perfusate’s composition on time. Monitoring can be performed by the assessment of the viability criteria used for short-term NMP, but new variables are likely required for long-term monitoring and assessment.

The current viability criteria which are often used for hepatocellular viability include lactate clearance, pH maintenance, glucose metabolism, bile production, perfusion flows, and transaminase levels. For cholangiocellular viability, bile composition, including absolute values of pH, bicarbonate, and glucose or their corresponding bile-to-perfusate ratios are used (83, 113–115). These viability criteria can potentially also be used for long-term monitoring of the liver during perfusion.

For long-term NMP, Eshmuninov et al. (15) used the existing viability criteria, but also proposed new criteria, such as hepatic artery response to vasoactive agents, the liver’s response to pancreatic hormones, a decline of inflammation markers (interleukin-6, interleukin-10), and distribution of the radioactive tracer 18-fluorodeoxyglucose imaged with positron emission tomography combined with computed tomography. Other techniques that are currently used in clinical practice may be adapted to monitor the liver function during NMP, such as the LiMax test (maximal enzymatic liver function capacity), which uses the metabolization of 13 C-methacetin, and is an independent predictor of initial dysfunction (116). In addition, the production of coagulation and complement factors may need to be monitored (58, 63, 72). For example, the coagulation state of the perfusate can be monitored by D-dimer and international normalized ratio measurements. The production of complement factors, such as C1, C3, C3a, and C5b-9 can be measured by enzyme-linked immunosorbent assays (117).

### DISCUSSION AND CONCLUSIONS

To achieve long-term liver MP preservation, a device should be designed to mimic key components of the human body, providing the liver with a stable and close-to-physiological environment. Because of the complexity of the
human body, this is not easily achieved. Some elements might be of more importance than others. For instance, the long-term oxygenators or artificial kidney have already shown to be an essential element to maintain a physiological environment. In addition, nutrients and hormones, diaphragm movement, and sterility have shown their necessity in long-term MP. The kind of pumps and tubing, the type of oxygen carrier, reestablishing denervation, a leukocyte filter, a change in anticoagulation or the supplementation of other additives might be considered less important, however still impact the perfusion. The automation of the machine itself may not appear important for the liver, but will cause fewer human errors and makes the machine more user friendly. However, aspects that may not be noticeable or important in short-term perfusions can easily grow to become significant issues over several days, and so a detailed and thoroughly considered approach is needed. To make a system capable of issues over several days, and so a detailed and thoroughly considered approach is needed.

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used in this manuscript.

drafted the manuscript; B.L. prepared the

2. [10.1007/s41586-018-0047-9]

B.L., A.M.T., T.L, M.W.N.N., R.J.P., and V.E.d.M. all approved the

M.W.N.N., R.J.P., and V.E.d.M. edited and revised the manuscript; B.L., A.M.T., T.L, M.W.N.N., R.J.P., and V.E.d.M. all approved the

that the idea and supervised the project; B.L. drafted the manuscript; B.L. prepared the figures. B.L., A.M.T., T.L., M.W.N.N., R.J.P., and V.E.d.M. edited and revised the manuscript; B.L., A.M.T., T.L, M.W.N.N., R.J.P., and V.E.d.M. all approved the final version of the manuscript.

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